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13. ABSTRACT (Maximum 200 Words) Breast cancers in humans often grow slowly or even remain undetectable for long periods of time only to reappear in discreet stages as progressively more malignant tumors. Recently, studies in both human cancers and experimental cancers in animals have established that cancers become progressively more aggressive in incremental steps that result from genetic mutations or "switches" in the tumor cells themselves. We have found that the two growth/differentiation promoting cytokines pleiotrophin (PTN) and midkine (MK) act as "switches" when introduced into breast cancer cells to stimulate more aggressive growth and induce new intratumor blood vessel formation, ie, an "angiogenic switch." Different studies have found constitutive expression of either the PTN or MK genes in over 50% of human breast cancers, suggesting our data is very important and relevant to human breast cancer. We now plan to pursue the mechanism of PTN signaling in both MMTV driven pleiotrophin gain of function mice and "knock-out" pleiotrophin mice developed in the laboratory and the mechanisms of downstream PTN signaling with different "chip technology"-driven strategies available to us in the laboratory.				
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BACKGROUND

What are the Mechanisms of "Tumor Promotion", of the "The Angiogenic Switch?"

Premalignant cells evolve to highly aggressive malignant phenotypes through sequential acquisition of genetic and epigenetic "switches" initiated by mutations that deregulate critical signaling pathways in the control of cell growth, the cell cycle, and programmed cell death [1]. Switches also are initiated when mutations influence the stromal microenvironment in ways to better support the malignant cells to grow, invade locally, and metastasize to distant sites [2, 3].

A uniquely important switch initiates tumor angiogenesis within the stromal microenvironment. It is an essential switch in the progression of malignant tumors since a vascular supply is essential for nutrients and adequate oxygenation and for tumors to grow beyond ~2mm in diameter and thus to exit "dormancy." The initiation of tumor angiogenesis and the tumor phenotype to a higher stage of malignancy is known as the "angiogenic switch". Currently, evidence favors the view that disruption of the normal steady state balance of the positive and inhibitory angiogenic pathways through "cooperative" sequential mutations is the mechanism of the angiogenic switch. However, the identity of the mutations and the temporal sequence of the different mutations in the malignant cell that lead to these more aggressive tumor phenotypes remains to be established [1, 4-8].

Are there properties of PTN in vitro that if signaled by an activated endogenous Ptn gene in the premalignant cell are likely to initiate an angiogenic switch?

Pleiotrophin (PTN the protein, *Ptn* the gene) is a lysine rich, highly basic developmentally regulated heparin binding cytokine of 168 amino acids (including a 32 amino signal peptide). Pleiotrophin is extremely highly conserved in bovine, rat, mouse, human and species [9]. We first purified PTN from bovine uterus as heparin binding growth factor (HBGF) 8[9, 10]. Pleiotrophin was also purified from neonatal rat brain as Hb GAM [11]. The *Ptn* cDNA encodes a protein with nearly 50% identity in amino acid sequence and with perfect conservation of disulfide bonds with another developmentally regulated cytokine, midkine (MK the protein, *Mk* the gene) and, together, PTN and MK are designated the PTN growth/differentiation factor family.

Many of the properties of PTN and other evidence is available to support the potential of PTN to initiate an angiogenic switch and a tumor phenotype of higher malignancy when the endogenous *Ptn* gene is activated in premalignant cells. Pleiotrophin stimulates proliferation of fibroblasts, [9, 10] endothelial cells [12-14], and epithelial cells [13, 15] in culture and initiates tube formation of endothelial cells in culture [16] and glial progenitor cells to enter the oligodendrocyte lineage specific differentiation pathway [17, 18]. Pleiotrophin has a

signal peptide and thus is readily secreted to cells into the extracellular milieu and is active on the different cells known to be in the tumor microenvironment. The *Ptn* gene is differentially expressed during development but minimally expressed in adults. However, *Ptn* gene expression is upregulated in injured tissue, such as endothelial cells and the macrophages and microglia surrounding new blood vessels of ischemia injured tissues [19].

What evidence supports Pleiotrophin (PTN the protein, Ptn the gene) as a putative target of activating mutations in premalignant cells? What are the consequences of an activated Ptn gene in the malignant cell?

The importance of pleiotrophin (PTN the protein, *Ptn* the gene) in malignant cell growth was first established when introduction of the exogenous *Ptn* gene into NIH 3T3 and NRK cells led to morphological transformation, anchorage independent growth, and subcutaneous tumor formation in the nude mouse (Chauhan, et al).

Subsequently, it was demonstrated that the minimally transformed (or "pre-malignant") SW 13 cells into which the exogenous *Ptn* gene was introduced were found to develop rapidly growing tumors in the flanks of athymic nude mice [20], establishing that expression of an ectopic PTN gene alone initiates a "switch" of the SW 13 cells to a more highly malignant phenotype. Furthermore, the consequence of an activated *Ptn* gene in MDA-MB-231 cells derived from a highly malignant breast cancer is a major increase in their malignant phenotype, making clear that an activated *Ptn* gene has the capacity to initiate a switch to more aggressive malignant phenotypes. In previously described experiments [21], it was shown when a dominant negative cDNA encoding a truncated PTN designed to hetero-dimerize and thus to inactivate the endogenous *Ptn* gene product during processing was introduced into the human breast cancer MDA-MB-231 cells, the MDA-MB-231 cells with constitutive high level expression of the endogenous *Ptn* gene were no longer transformed; the dominant negative *Ptn* gene reversed the ability of the MDA-231 cells to form plaques or colonies in soft agar and to form tumors in the athymic nude mouse [21], see data below.

Dominant negative PTN (HBC *Ptn*-1, HBC *Ptn*-2) reverses colony formation of MDA-MB-231 cell lines in soft agar assay and prevents tumor formation in the nude mouse

Stably transfected	Colonies in soft agar assay	tumor size
MDA-MB-231 cell line	(percent of cells seeded)	(mm ²)
HBC <i>Ptn</i> -1	~0.8	10 ± 4
HBC <i>Ptn</i> -2	~0.75	15 ± 8
HBC vector-1	~8.5	65 ± 12

HBC vector-2	~8.2	70 ± 8
HBC vector-3	~7.9	68 ± 10
HBC vector-4	~8.1	61 ± 14

This study, together with studies of Wellstein et.al. demonstrating the transformed phenotype of human melanoma cells (WM852) is suppressed by *Ptn*-targeted ribozymes [22], provide striking support for the conclusion that premalignant breast cancer, melanoma, and perhaps other premalignant cells that acquire an activated *Ptn* gene through mutation initiate a dramatic increase in their malignant phenotype.

Is an activated Ptn gene frequent in other human malignancies?

Pleiotrophin gene expression has been described in neuroblastoma, glioblastoma, prostate cancer, lung cancer, Wilms' tumor, many breast cancers and, in one study about one-fourth of over 40 human cell lines of different origins [13, 21-28]. In each of these studies, *Ptn* gene expression is low or not detected at all in the normal tissues from which the malignant cells were derived and, in each cell line derived from these tumors that has been tested, endogenous *Ptn* gene expression is constitutive and thus deregulated.

The data from experiments presented subsequently not only strongly support the hypothesis that an activated *Ptn* gene alone is sufficient to signal an angiogenic switch and a highly malignant phenotype in premalignant cells but furthermore define a mechanism to account for the PTN phenotype of the more malignant cells expressing cells.

ACHIEVEMENT: PTN contains an independent angiogenic domain: relationship to the "Angiogenic Switch".

We examined tumors in the nude mouse derived from NIH 3T3 cells transformed by mutant PTN residues 41-136 coupled with the endogenous signal peptide and found they were considerably more vascular than tumors derived from NIH 3T3 cells transformed by PTN 1-64. To pursue the significance of this finding, we repeated the experiments with wt (full length) PTN 1-136, mutant (N-terminal) PTN 1-64, and mutant (C-

terminal) PTN 69-136 transfected NIH 3T3 cells. We also established SW13 cell lines that constitutively expressed each of these proteins. SW13 cells are derived from a human adrenal carcinoma, lack aggressive growth, develop only very small tumors in the nude mouse, and have insignificant tumor angiogenesis. The SW13 cell is ideal to test PTN and its domains in tumor promotion assays since increases in the aggressiveness of these cells readily measured and the SW13 cell can be readily defined as "pre-malignant."

As shown below, the results were dramatic; SW 13 cells transfected with PTN 1-64 and PTN 1-136 implanted in the nude mouse developed marked increases in tumor size and intratumor microvessel formation (a measure of blood vessel density) in comparison to SW 13 cells transfected with vector alone; the increase in tumor size and in intratumor microvessel density in SW13 cells induced by PTN 1-136 was greater than PTN 1-64, indicating the possibility that a domain within PTN 65-136 is a tumor promoter and promotes angiogenesis. To directly test this possibility, PTN 69-136 was tested in both NIH 3T3 cells and in SW13 cells. Remarkably, the rate of tumor growth and neovascularization in SW13 cells was markedly enhanced, but, as previously demonstrated, PTN 69-136 failed to transform NIH 3T3 cells.

The results establish that PTN 69-136 contains a domain that significantly enhances new blood vessel formation in an already transformed "pre-malignant" cell line. However, PTN 69-136 lacks the ability alone to transform. The results establish that residues 69-136 contains a strongly angiogenic domain that is structurally and functionally independent of the transforming domain of PTN. They establish that PTN residues 69-136 cannot function in a non-transformed cell line but requires an activated transformation pathway supplied by the SW13 cell with which to cooperate. Constitutive expression of PTN 69-136 thus "cooperates" with an already activated, genetically stable, and presumably transformation-dependent pathway in the transformed SW13 cells to signal tumor angiogenesis. It is not detectably functional in the non-transformed NIH 3T3 cells. The data are summarized below.

	<u>Tumor (mm²)</u>		<u>Intratumor Microvessel Density IMD (#)</u>	
	<u>NIH 3T3</u>	<u>SW13</u>	<u>NIH 3T3</u>	<u>SW 13</u>
PAGE 103 vector alone	0	12 mm ²	0	~5
PTN 1-136	145 mm ²	60 mm ²	~55	~30
PTN 1-64	108 mm ²	44 mm ²	~44	~7

ACHIEVEMENT: Pleiotrophin Strikingly increases rate of growth of MCF-7 cell subcutaneous tumors.

The MCF 7 cell is derived from a human breast cancer and is widely used to model the behavior of breast cancer cells since it expresses the estrogen receptor and is responsive to estrogens when implanted in mammary fat pads of nude mice. In humans with breast cancer, estrogen responsive tumors after time assume a more aggressive malignant phenotype characterized by a more malignant cell phenotype morphologically and the onset of tumor angiogenesis, metastasis, refractoriness to therapy, and a poor prognosis for host survival. These “switches” to the more aggressive tumor phenotype (see Background, above) are often associated with a loss of the estrogen receptor in the tumor cells. The “switch” to the more aggressive growth phenotype with the onset of tumor angiogenesis has been demonstrated in humans by morphological criteria and in experimental mice in which targeted oncogenes have been introduced. Since the switch was observed in successive generations of mice, a genetic basis for the switch was established, and thus the switch is now viewed as the consequence of a mutation in a “pre-malignant” cell.

In these experiments, we attempted to discover a basis for the “switch” in mammary cancer using as a model the MCS 7 cells implanted subcutaneously in the flanks of nude mice.

*Materials and Methods*Expression Constructs:

The full-length human *Ptn* cDNA encodes a 168 amino acid protein that includes a 32 amino acid signal peptide. The cDNAs encoding the N-terminal (amino acid residues 1-64) and C-terminal (amino acid residues 69-136) domains of PTN and the full length PTN (amino acid residues 1-136) coupled with the endogenous signal peptide were cloned into the pAGE 103 vector as previously described [29].

Cells, cell culture, DNA transfection:

Human MCF 7 cells at early passage were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. DNA transfections were performed by calcium phosphate co-precipitation as described [30]. The transfectants were selected with G418 for three weeks. In each case, colonies were clonally selected, and the clonal cell lines were established, expanded, and

confirmed by Northern and Western analysis (data not shown); clones with high-level expression of exogenous genes were retained for further study.

Northern blots:

($\alpha^{32}\text{P}$)-dCTP labeled-*Ptn* cDNA was used for overnight hybridization with total cellular RNA. Blots were washed with 0.1 X SSC and 0.1% SDS at room temperature and exposed to X-ray film with intensifying screens at -70°C as previously described [25].

Western blot analysis:

Cloned transfectants that harbor the expression vectors described above or an empty vector (control) were lysed in 0.8 ml of cell lysis buffer (10 mM Hepes, pH 7.2, 142.5 mM KCl, 1 mM EGTA, 5 mM MgCl_2 , and 0.5% NP40) per 100 mm dish and centrifuged. The supernatants were incubated with 25 μl of heparin Sepharose beads (Pharmacia) at 4°C for 24 hours, washed x3 with Tris buffered saline containing 0.1% Tween 20 (TBST, pH 7.35), and eluted with 15 - 30 μl SDS gel loading buffer. The eluates were separated by 12.5% SDS-PAGE, transferred to immobilon membranes (Millipore), immunoblotted with chicken anti-human PTN, and visualized by the ECL system (Amersham).

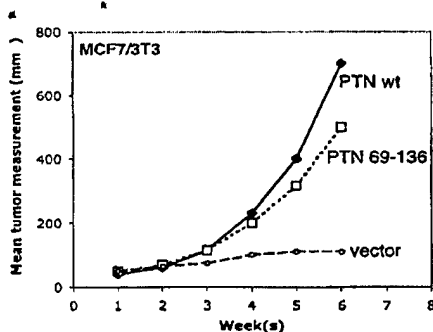
Tumor Progression, Subcutaneous Tumor Formation in Nude Mouse.

The MCF 7 cells are derived from a human breast cancer and form few if any small tumors in flanks of nude mice when implanted subcutaneously (a "hostile environment"). In this context, the cells are "pre-malignant."

Female athymic nude mice (Ner nu/nu; Harlan Sprague Dawley, Indianapolis, IN) at 6 weeks of age were injected subcutaneously in each flank with 2×10^6 cells MCF-7 and 2×10^6 NIH 3T3 cells from pooled clonal cell lines. Tumor size was checked daily beginning 10 days after injection. At 6 weeks, selected animals were sacrificed and tumor size was estimated as the product of two diameters at right angles to each other [30].

Representative tumors from each of the established cell lines was examined by hematoxylin and eosin (H&E) staining and analyzed for intratumor microvessel density as described below.

Intratumor Microvessel Density:



Intratumor microvessel density (IMD) is a measure of the relative density of tumor angiogenesis in sections of tumor tissue prepared from tissues fixed in 10% buffered formalin and embedded in paraffin. Sections were deparaffinized and rehydrated by passage

through decreasing concentrations of ethanol, incubated for 30 min in 0.3% hydrogen peroxide solution (Sigma), washed twice in PBS (Sigma), and blocked with a 1:66 dilution of normal horse serum (Vector Laboratories) for 30 min. The blocking sera were removed and replaced by a 1:200 dilution of rat anti-CD31 (PECAM-1) monoclonal antibodies (MEC 13.3, Pharmingen) in blocking serum, incubated overnight at 4°C in a humidified chamber, washed x2 with PBS for 5 min, and incubated with a 1:200 dilution of (secondary) peroxidase-coupled rabbit anti-rat (mouse adsorbed) serum (Vector Laboratories) for 30 min. Immune complexes were highlighted with diaminobenzidine (Sigma) and counterstained using Harris hematoxylin (Lerner Laboratories, New Haven, CT), dehydrated, and mounted. Stained capillaries were counted at x400 magnification by independent, uninformed investigators and analyzed by statistical analysis of test and control groups. H and E - stained sections were used to select representative areas of the invasive tumor components and IMD was determined as described by Weidner [31].

Results:

These data presented below, make clear that *wt* and PTN residues 69-136 enable MCF-7 cells to grow and to grow aggressively as subcutaneous tumors in nude mice whereas the vector alone containing MCF-7 cells grow little if at all in this site. The growth of PTN and PTN mutant cells containing MCF-7 requires a microenvironment supplied by NIH 3T3 cells. Without NIH 3T3 cells, the subcutaneous tumors grow but the rate of growth is slow.

ACHIEVEMENT: Demonstration at separate domains of Midkine enhance strikingly the growth of MCF-7 cells as subcutaneous tumors in the nude mouse.

Research Design & Methods: Detailed above and below, Methods specific to this Achievement

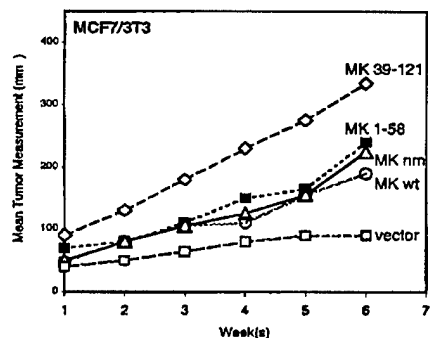
Soft-agar assay and tumor formation in nude mice:

For tumor formation, female athymic nude mice (Ner nu/nu; Harlan Sprague Dawley, Indianapolis, IN) will be injected subcutaneously in each flank with 2×10^6 cells from the pooled, clonally selected cell lines expressing the cDNA's to encode MK and the MK mutant proteins illustrated below and with equal numbers (2×10^6) of NIH 3T3 cells. Tumor size was estimated daily, starting at 10 days following injection. After 6 weeks, selected animals were sacrificed and tumor size was estimated by measuring two diameters of right angles as described previously [30] and one tumor representing each established cell line was for intratumor microvessel density.

Human MK is encoded by a 429 bP cDNA fragment and is composed of 143 amino acids including a 22 amino acid signal peptide. Human MK gene contains 5 exons, but exons 2-5 encode MK protein primary amino acid sequence and exon 1 of MK, like PTN, compromise only 5' untranslated region. The mature human MK is about 14 KD in SDS-PAGE gels. The cDNA fragments encoding the MK mutants will be generated using PCR methodology and was cloned into the Hind/Xho1 sites of the pAGE 103 new vector by fusing to the Ig kappa signal recognition sequence at the N-terminus and fusion to the c-myc and 6x polyhistidine tags at the C-terminus.

RESULTS:

These data presented below, make clear that *wt* MK and MK domains 39-121, MK 1-58, MK nm promote the growth of MCF-7 cells as subcutaneous tumors in the nude mouse. Although the results are not as dramatic as those seen with PTN, it is clear that MK expression in the MCF-7 cell enhances or promotes its ability to grow as a subcutaneous tumor in the nude mouse when implanted with equal amounts of NIH 3T3 cells. Each of these cells grew to a limited degree (data not shown) when expressed without NIH 3T3 cells, in each case more so than vector alone transformed cells. These data thus establish that a deregulated *Mk* gene in human breast cancer cells also promotes their growth as aggressive to a more aggressive malignant phenotype. Importantly, MK nm stands for the natural mutant that has been found previously in breast, colon and prostate cancer. This mutant is the result of alternative splicing and this result thus supports the suggestion that this naturally occurring alternatively spliced form of MK when acquired during the course of tumor progression establishes a more aggressive growth phenotype in human breast cancer. These data also make clear different signaling domains MK signal independently of each other.



ACHIEVEMENT: PTN Signals a “Scirrhous Phenotype” or “Epithelial Island” Phenotype both *in vitro* and *in vivo*: A Model of the Scirrhous Breast Cancer Phenotype.

FIGURE 1: MCF-7 CELLS IN CULTURE: Left side, MCF-7 cells, mock transfection. Right side, MCF-7 cells with high level expression *Ptn* gene. Upper Row, logarithmic growth. Bottom Row, at confluence.

RESULTS: No evidence of epithelial island formation.

Methods

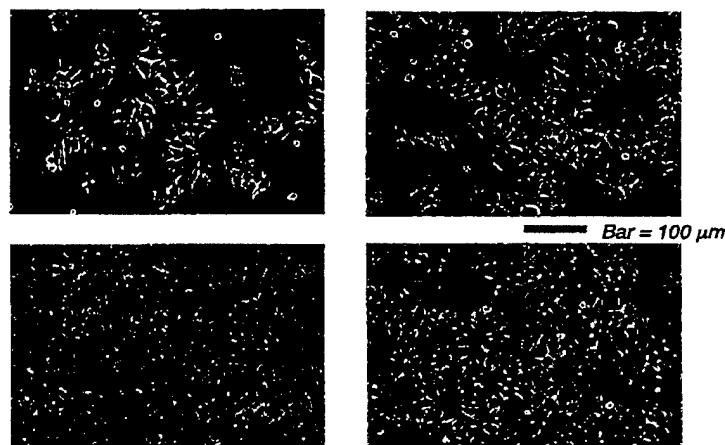


FIGURE 2: CO-CULTURE, 1:1 RATIO, MCF-7 CELLS: NIH 3T3 CELLS

Left side, MCF-7 cells, mock transfection. Right side, MCF 7 cells with high-level expression *Ptn* gene. Top Row, late logarithmic growth. Bottom row, at confluence.

RESULTS: At confluence, epithelial islands are sharply demarcated in *Ptn* expressing MCF-7 cells.

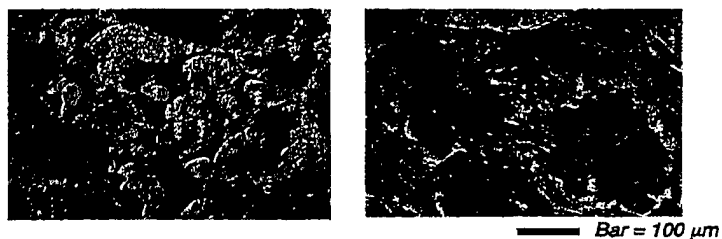


FIGURE 3: CO-CULTURE, 1:1 RATIO, MCF-7

CELLS: NIH 3T3 CELLS

Left side, MCF-7 cells, mock transfection. Right side, MCF 7 cells with high-level expression *Ptn* gene. Top

Row, late logarithmic growth. Bottom row, at confluence.

RESULTS: At confluence, epithelial islands are sharply demarcated in *Ptn* expressing MCF-7 cells.

Hematoxylin and Eosin Stain

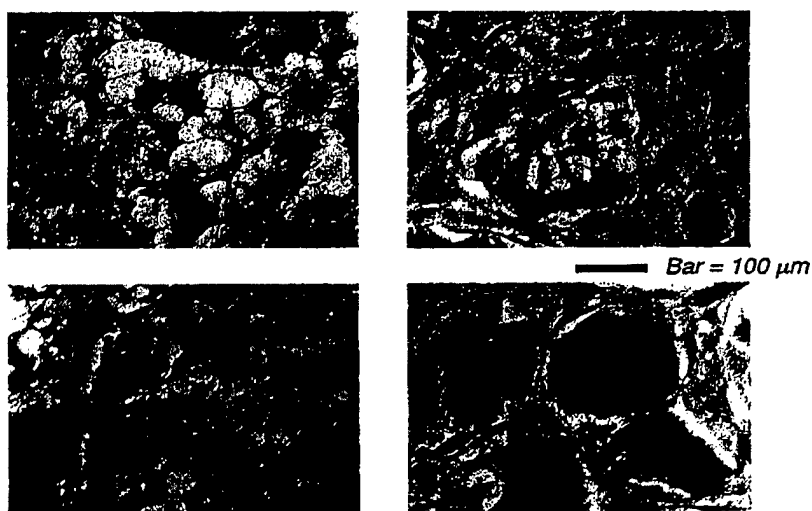


FIGURE 4: CO-CULTURE at DIFFERENT RATIOS at MCF-7 cells:NIH 3T3 Cells, left to right 1:2, 1:1, 1:2. Demarcation of epithelial islands is sharp but epithelial islands vary in size depending on ratio of MCF-7 cells to NIH 3T3 cells.

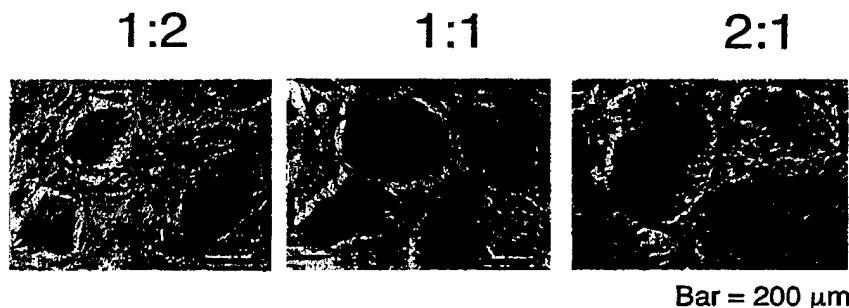
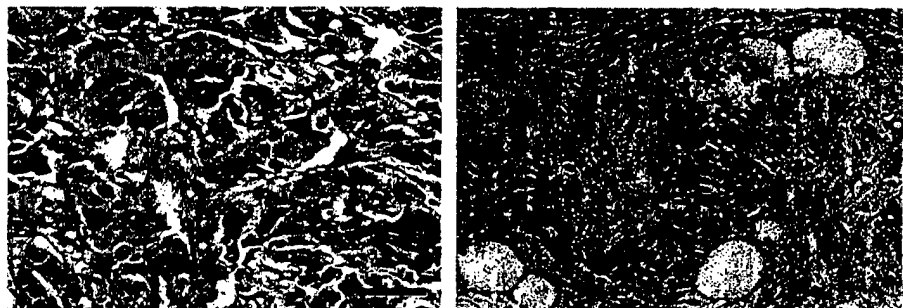
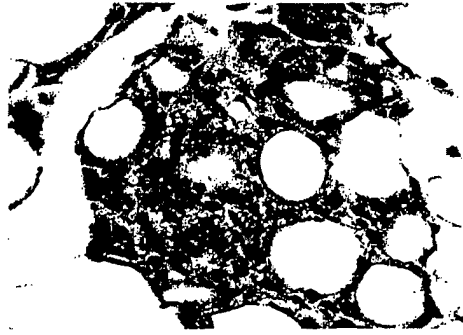
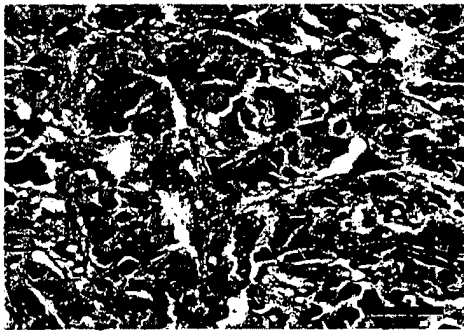


FIGURE 5: MCF-7 CELL SUBCUTANEOUS TUMORS in NUDE MICE at TWO WEEKS: Upper Left, Lower left are duplicate figures. Co-injection at 1:1 ratio, MCF-7 high-level expression *Ptn* gene: NIH 3T3 cells. Upper Right, Co-injection mock transfected MCF-7: NIH 3T3 cells. Bottom Right, Mock Infected MCF-7 cells alone.

RESULTS: Only MCF-7 cells with high-level expression *Ptn* gene with NIH 3T3 cells form aggressively growing tumors.



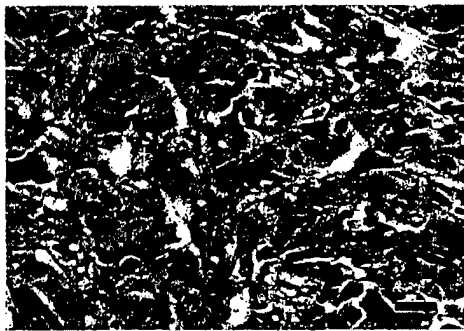
H&E stain, Bar = 100 μ m



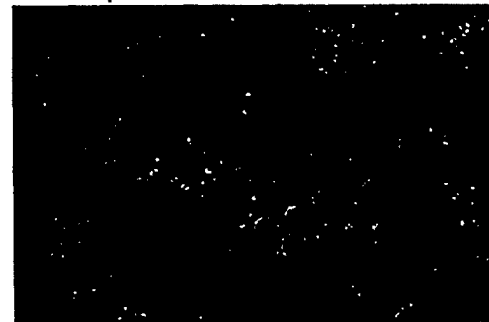
H&E stain, Bar = 100 μ m

FIGURE 6: CO-INJECTION into the FLANKS OF NUDE MICE (Left Side), CO-CULTURE(Right Side) of MCF-7 CELLS with HIGH LEVEL EXPRESSION *Ptn*. NIH 3T3 CELLS are equal numbers. Left Side, MCF cell subcutaneous tumors, Top: Hematoxylin and Eosin Stained Section. Bottom, Frozen Section, *In Situ* Hybridization, anti-sense RNA *Ptn* probe. Right Side, Bottom, Co-Culture MCF-7 Cells with High Level Expression *Ptn* :NIH 3T3 Cells, Nomarski Optics.

RESULTS: Epithelial Islands are seen in all views High level PTN expression is shown by in situ hybridization of MCF-7 Cells.



Bar = 50 μ m



ACHIEVEMENT: Progress in PTN gain-of-function breast cancer transgenic mice

The mice model we have:

- 1, FVB-MMTV-Neu, 2 breeding pairs (both are homozygotes). DOB is 9-23-03; 20 female homozygotes and 8 male homozygotes, DOB is 11-25-03.
- 2, FVB-MMTV-Wnt1, 1 breeding pair (male hemizygote X wildtype female), DOB is 11-2-03.
- 3, FVB-p53KO. 4 breeding pairs (+/- X +/-), DOB is 12-25-03.
- 4, FVB-PyMT. 1 breeding pair (male T/+ with female +/+), DOB is 5/14/04.
- 5, FVB-MMTV-PTN-IRES-GFP.

We have 8 positive founder mice. All of them are female, DOB is 8-12-03.

After mating these mice with inbred FVB mice, we obtained 56 F1 mice (DOB is from 1-06-04 to 1-12-04). Among these, 9 mice from 5 different founders are positive. After breeding with FVB mice we obtained 12 F2 mice from 3 different F1 mice. It is planned to pick up 2 mice(one male and one female) from each founder to confirm PTN's expression (Northern blot and in situ hybridization) . The positive mice will be mated with the other gain of function mouse breast cancer predisposition mice.

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